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THE PIG MYOGENIN GENE AND METHOD TO IDENTIFY POLYMORPHISMS RELATED TO MUSCLE GROWTH

The invention relates to the field of molecular biology as well as to the field of breeding methods for farm animals, in particular pigs. In particular the invention relates to the use of diagnostic methods derived from the field of molecular biology in breeding programmes that select animals on production traits that improve their breeding value.

By selecting animals on their breeding value calculated mainly from phenotypic measurements of production traits, breeding has greatly improved the genotype for production traits of livestock animals. Thus, traditionally, breeding programmes have selected for phenotypic characteristics of animals. However, more recently selection for genotypic characteristics that are associated with improved production traits have gained interest in the field. Selection for phenotypic characteristics entails mainly selection of the offspring or siblings or other relatives of the animals to be selected whereas selection of specific genotypic characteristics allows for earlier and specific detection of animals of interest.

Within methods that select on specific genotypic characteristics, one may distinguish between methods that detect genetic variation in genes or quantitative trait loci that are merely associated with production traits of animals and methods that detect genetic variation in functional genes that directly influence those production traits. One of the former methods is a marker assisted selection wherein polymorphisms in markers identified in a random manner are associated with production traits.

For instance, meat production is closely linked to embryonic muscle formation. Biologically, production is concentrated in defined tissues of the animal, e.g. muscle

tissue for lean meat production. In breeding programmes for optimizing porcine lean meat production, various levels of selection pressure have been applied to different tissues (i.e. muscle, fat and bone).

Muscles are complex tissues composed of a number of different cell types, e.g. myocytes (the most predominant) consisting of myofibres and satellite cells, intramuscular adipocytes, fibroblasts, endothelial cells, neurocytes, etc. Handel and Stickland (1984, 1988) showed that the number of myofibres present at birth determines the maximal lean meat growth capacity of pigs. Double-muscled cattle show a higher number of prenatally developed myofibres than other cattle (Swatland and Kiefer 1974; Hanset et al., 1982), which suggests that lean meat production capacity is determined by the embryonic development of myocyte number.

Myogenesis is a complex, multistep process that chronologically involves:

(1) Progenitor cell determination to the myogenic lineage.

(2) Migration of myogenic stem cells (myoblasts) to appropriate locations in the early embryo.

(3) Proliferation of myoblasts and non-myogenic muscle-tissue cells.

(4) Terminal myocyte differentiation (i.e. fusion of myoblasts) and expression and organization of specific gene products active only in terminally differentiated muscle cells.

(5) Maintenance of the terminal differentiated state and modulation of myofibres in various myofibre types in response to age and physiological cues (Edgerton and Roy, 1991; Funk et al., 1991; Gunning and Hardeman, 1991; Olson, 1992).

A model based on the action of the MyoD gene-family describes a mechanism for the genetic regulation of myogenesis. There are four members of this family in vertebrates, MyoD (also called Myf-3), myogenin (Myf-4), Myf-5 and MRF4 (Myf-6, herculin). A number of recent reviews summarizes in detail the existing knowledge of the structure of the genes, the MyoD-myogenesis model and the activation of muscle tissue-specific genes by the MyoD genes (for reviews see above).

MyoD proteins are expressed specifically in muscle tissue where they act as tissue-specific transcription factors. *In vitro* they are active after formation of dimer complexes with proteins of the ubiquitously expressed E2A gene. The complex binds to specific transcription regulatory sequences of muscle-specific genes called enhancer regions in the promotors, thereby activating expression of the tissue- and developmental stage-specific genes like muscle-specific actin, tropomyosin and titin (reviewed in Olson, 1990; Weintraub et al., 1991; Lyons and Buckingham, 1992).

Once activated, each member of the MyoD gene family can both positively autoregulate its own expression and regulate the expression of other MyoD genes in differentiating in vitro muscle cell cultures, thereby continuing the differentiation pathway. Thus, once the pathway is activated, myogenesis continues until terminal differentiation is established.

Determinated cells (myoblasts) are able to migrate (step (2) of the myogenesis pathway) and profilerate (step (3)); Olson, 1990, 1992). Irreversible terminal differentiation (step (4)) is induced by fusion of the myoblasts into multinucleated myofibres. The fusion is induced by the activation of the myogenin (Myf-4) gene in myoblasts (Olson, 1990, step (4)).

The research for genetic variation within the MyoD genes is already underway in a number of laboratories. Recently the first polymorphism was reported in the myf-4 gene in pigs (Ernst et al., 1993). Allele freqencies differed between different pig breeds. It is still unclear where in the gene this polymorphism is located and whether this polymorphism could be related to different muscle growth potentials and be used as a marker within selection lines of one breed.

Myogenin is the only myf gene expressed in all skeletal muscle cell lines (Wright et al., 1989; Edmondson and Olson, 1989). Knock out experiments in mice have shown that this protein fulfils an essential function in muscle differentiation by regulating the onset of myoblast fusion and the formation of

functional muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993).

RFLPs have been described for the mouse MyoD gene (Kay et al., 1993), the bovine myf-S gene (Dean et al., 1993) and the pig myogenin (Ernst et al, 1993) and myf-6 genes (Ernst et al., 1994). One MyoD allele in mouse seems to be associated with the increased efficiency of muscle regeneration of the SJL/J strain.

The present invention provides among others an isolated or recombinant pig myogenin gene specific nucleic acid molecule or pig myogenin gene specific fragments thereof comprising or hybridising to the nucleotide sequence of Fig. 1 or its complementary sequence or the RNA equivalents thereof.

Myogenesis could be an important determinant of the number of muscle fibers present at birth and thus influence production traits such as birth weight or quantitative lean meat production, or other traits of interest. With the present invention, the genetic variation within the myogenin genes in breeding populations can now be revealed and analysed for association of the effects of each of the alleles with production traits of interest. Genetic variation within the myogenin gene could also be associated with different regulation of expression. Since myogenin regulates the moment in time at which differentiation proceeds with fusion of existing myoblasts and the end of myoblast proliferation, delayed or changed expression levels can influence the number of muscle cells formed. With the present invention, the genetic variation within the myogenin genes with respect to variation in regulation of expression can now be revealed and analysed for association with production traits.

The present invention further provides a method to generate via recombinant DNA techniques an animal, such as small laboratory animals or farm animals, i.e. a pig, with additional genetic material originating from the pig myogenin gene. Such animals may than encode wanted alleles of the pig myogenin gene and constitutively or transiently express allelic

proteins or fragments thereof that enhance the production characteristics of those animals.

The invention further provides methods to generate proteins or (poly)peptides comprising various allelic proteins or fragments thereof derived from the pig myogenin gene. Such peptides, or antibodies specifically directed against such peptides, may be used to influence production traits in the live animal, but may also be used in cell-culture systems *in vitro*. Such peptides, or antibodies specifically directed against such peptides, may also be used in diagnostic test systems to select animals that express wanted forms of allelic proteins or fragments thereof encoded by the pig myogenin gene.

The invention further provides methods localising, identifying or marking genes or alleles or quantitative trait loci, in particular of the pig myogenin gene, in samples, in particular biological samples, cells or tissues, such as but not limited to hair, skin or blood, of pigs, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci of pigs. Since marker assisted selection of pigs is frequentially based upon genetic variation that exists within functional genes that influence a production trait directly, i.e. genes such as the pig myogenin gene that controls myogenesis, one of the methods that the invention provides is a method that identifies or marks such genes and that can distinguish between characteristics of alleles of those genes which characteristics serve as markers in selection programmes for animals with specific versions of those genes that are directly linked with improved production traits.

The invention further provides a method wherein polymorphic restriction sites within functional genes and thus different alleles of those genes are identified by allowing for specific amplification of genomic fragments of those genes, in particular by allowing for specific amplification of fragments of the myogenin gene. Amplification methods are well known in the art, the best known being PCR. A short description of the PCR used herein is given in the experimental part. Other

primers, enzymes and conditions can of course be applied. After amplification a suitable method of identifying wanted alleles is a restriction endonuclease treatment. A suitable restriction enzym for pig myogenin alleles is *MspI* but others may also be used. By these methods large numbers of pigs can be rapidly genotyped for studies in which genotypic variation can be associated with growth characteristics and other production traits of pigs

However, there are many other methods identifying polymorphisms in alleles, both at the nucleic (DNA/RNA) level and at the product (protein) level. In particular at the protein level there are many possibilities using immunoassays, whereas at he nucleic acid levels there are many assays which all include some kind of hybridisation step of for instance primers or labelled nuclei acids. A very good possibility would be mismatch PCR. Primers to be used in the invention can be identified by the person skilled in the art, the sets given in the experimental part are for illustrative purposes only.

Furthermore, the methods according to the invention can be developed into diagnostic assays or kits by which selection of pigs with alleles of interest can be performed in routine screening protocols employed in breeding programmes. With such protocols better results of selection can be expected when genes responsible for regulation of commercially interesting body tissues can be rapidly identified and controlled.

EXPERIMENTAL

(a) Amplification, cloning and sequence analysis of the porcine myogenin gene.

In order to study genetic variation at the porcine myogenin gene locus, specific PCRs were developed (PCR 1 and 2, Table I). Primers were based on an alignment of human (Salminen et al., 1991) and mouse myogenin sequences (Edmondson et al., 1992). Fragments of 2099 and 442 bp respectively, covering the complete coding sequences and two introns, were obtained by PCR

amplification on pig genomic DNA. These fragments were cloned in pUC18 and sequenced (Fig 1). Like the human and mouse genes, the pig myogenin gene has three exons. The first exon encodes the bHLH domains, which in men, mice and pigs have identical amino acid sequences. Exon 2 encodes 27 amino acids of the transactivation domain (Schwarz et al., 1992) and exon 3 the conserved C-terminal segment, common to the four Myf proteins (Fujisawa-Sehara et al., 1990). The amino acid sequence of pig myogenin is for 97% and 96% identical to the sequence of human and mouse Myogenin, respectively. The first pig intron is 785 bp long and is larger than the corresponding introns of mice and men. The second intron of 639 bp is larger than the mouse but smaller than the human second intron.

The myogenin promoter and 3' non coding sequences were isolated by screening of a porcine genomic library with a mixture of human myf cDNA fragments. Positive phages were purified and myogenin clones were selected by means of PCR 1 and PCR 3 (Table I). The *SacI*-fragments of two clones (A16, A17) were subcloned in pBS- and sequenced. This revealed 571 bp of the promoter sequences and 856 bp of the 3' untranslated region, containing a putative polyadenylation signal (AATAAA) at positions 3397 to 3402.

Analysis of the pig myogenin promoter for the presence of transcription factor binding consensus sequences revealed three E-box sequences (CANNTG) located at positions 508 to 513 (E1), 210 to 215 (E2) and 22 to 27 (E1). A myocyte-specific enhancer factor 2 (MEF-2) site (456 to 464), a nuclear factor 1 (NF-1) site (469 to 481) and a TATA-box sequence (494 to 498) were identified within 70 bp of the putative transcription start sites. Comparison of the pig sequence with the human and mouse sequences revealed more than 96% sequence identity within the proximal 160 bp upstream from the transcription start sites. This high conservation may be explained by the observation that this region specifies the correct spatial and temporal activation of the mouse myogenin gene during embryological development (Cheng et al., 1993; Yee and Rigby, 1993).

The E1-box is present in the myogenin genes of men, mice and pigs, whereas the E2 box is only conserved between the human and porcine genes. The E1-box has been demonstrated to be the only myogenin binding E-box sequence in the mouse promoter (Edmondson et al., 1992). Funk and Wright (1992) identified MEF-2 and NF-1 as stabilizing factors for the in vitro binding of myogenin to E-box sequences. The presence of binding sites for both factors in the highly conserved minimal proximal promoter sequences of the different myogenin genes indicates that these factors are involved in the regulation of the myogenin expression. Especially the presence of the MEF-2 site appears to be crucial for the correct embryological myogenin expression (Cheng et al., 1993; Yee and Rigby, 1993).

(b) Characterization of a CA-repeat at the myogenin gene locus

A CA-repeat sequence of variable length (19 and 22 repeats) has been identified by Edmondson et al. (1992) and Yee and Rigby (1993) between positions -603 and -560 in the mouse myogenin promoter. (CA)n-repeats, with n > 6, are the most abundant highly polymorphic dinucleotide repeats, present in mammalian genomes (Wintero et al., 1992) and they are extensively used as markers in association studies. Because of our interest in polymorphisms closely linked to the myf genes, we also examined the myogenin phage inserts with a (CA)25-repeat probe. This revealed the presence of a CA-repeat sequence at about 5 kb downstream of the TGA termination codon. Sequence analysis of this region demonstrated that the CA-repeat was interrupted by CCC stretches (sequence submitted to the EMBL/GenBank/DDBJ databases and available under accession number X89210). Since imperfect CA-repeats are generally not polymorphic (Wintero et al., 1992), we did not further examine this region.

(c) Characterization of a polymorphic *MspI* site at the 3' side of the porcine myogenin gene

The presence of a variable 4.2/4.9 kb *MspI* fragment at the myogenin gene locus has been previously described for different pig breeds by Ernst et al. (1993). Southern blot analysis of 105 unrelated pigs from 7 pig breeds with a probe specific to the 3' side of the myogenin gene (Fig 2a), and restriction mapping of myogenin subclones, enabled us to localize the described polymorphic *MspI* site at the 3' side of the gene and to sequence its flanking regions (sequence submitted to the EMBL/GenBank/DDBJ databases and available under accession number X89209). A PCR-RFLP test (PCR 4, Table 1) for detection of this polymorphic site was developed and used for genotyping the pigs of the panel (Fig. 2b).

All typed breeds except the Meishan, are polymorphic at the detected *MspI* site (Table II, columns 9, 10, 11). Comparison of the allele frequencies for the different pig breeds tested, shows that the Meishan population under investigation does not contain the 4.2 kb allele. Ernst et al. (1993) also found the 4.9 kb to be the predominant myogenin allele in Chinese pig breeds (Fengjing, Meishan, Minzu). However, this might be explained by the very small numbers (< 10) of founder animals of these Chinese pig populations in the US and in Europe (Brascamp, pers. comm.), as well as by the small numbers of animals tested. In Western breeds, the 4.2 kb allele seems to be more frequent, but the allelic distributions depend on breed and population. We found the 4.2 kb allele to be predominant in Duroc and Wild Pig breeds. Both alleles were equally present in Pietrain, Dutch Landrace (DL) and Hampshire, while the Great Yorkshire (GY) population tested was characterized by a high frequency of the 4.9 kb allele.

(d) Detection and characterization of two additional polymorphic *MspI* sites at the porcine myogenin gene locus

MspI digestion of PCR1 products of the 105 unrelated pigs revealed sequence variation at position 2394 in the second intron. Analysis of the PCR1-*MspI* patterns shows sequence variation near position 2394 for the Meishan, DL and Duroc breeds (Table II, columns 6, 7, 8). In the Meishan population only, the absence of the 2394 MspI site is coupled to the presence of a 1.8 kb *MspI* fragment on Southern blot hybridized to the PCR1 probe (Fig. 3a and b), while in all other breeds only a 2.3 kb *MspI* fragment is visualized. This implies that in Meishans a third variable *MspI* site is present in the promoter region of the myogenin gene. Remarkably, several attempts to amplify the concerned region with different primer combinations (e.g. PCR5, Table I) failed to generate a fragment for the Meishan specific allele (Fig. 3c). This suggests that additional sequence variation at the primer hybridization sites hinders proper amplification of the 1.8 kb allele or that a deletion rather than an additional *MspI* site causes the 1.8/2.3 kb polymorphism.

Autosomal Mendelian segregation of the 1.8 kb and 2.3 kb pig myogenin fragments and of the MspI polymorphism in the second intron was checked in 58 Meishan pigs from 4 two-generation families and in a DL heterozygous x homozygous cross (9 animals, data not shown). Analysis of the genotypes of the 105 unrelated pigs from 7 different breeds, and of the progeny of the DL cross, suggests the presence of at least 4 different myogenin alleles in pigs (Fig 4). The allele frequencies are breed dependent, with allele 4 being specific for the Meishan breed, allele 3 being only detected in a few animals of the DL and Duroc breeds and alleles 1 and 2 being the predominant alleles in the Western breeds. Large numbers of commercial European pigs will be genotyped in the near future by use of the PCR-RFLP tests for *MspI* sites a and b (Fig 4), and the resulting myogenin genotypes will be tested for association with growth characteristics.

(e) Chromosomal assignment of the porcine myogenin gene

Localization of the myogenin gene in the pig genome offers the possibility to use a limited set of microsatellite markers in association studies with growth characteristics. We analysed a panel of 21 pig/rodent somatic cell hybrids for the presence or absence of the pig myogenin gene by myogenin specific PCR 3 amplification (Table I) on genomic DNA. A pig myogenin fragment with a predicted size of 873 bp was amplified, together with the hamster or mouse fragment from the parental rodent cell line, of 650 and 600 bp respectively. The porcine PCR 3 fragment was further identified by *EcoRI* and *PvuII* digestion, generating fragments of respectively 313 + 560 bp and 219 + 654 bp.

The distribution pattern of specific signals in all hybrids was compared with the signals for all reference loci. The highest concordance for myogenin was 90% and the significant correlation value o was 0.81 for chromosome 9, indicating that myogenin maps to chromosome 9 (Table III). Myogenin has already been mapped by RFLP analysis to chromosome 9 (Archibald et al., 1995). We have confirmed these results by physical mapping with somatic cell hybrids. In human myogenin maps to chromosome 1q31-q41. Our result confirms a segment of conserved synteny between human chromosome 1 and porcine chromosome 9, which has been demonstrated by linkage mapping of CR2 (Johansson et al., 1994) and Zoo-FISH analysis (Rettenberger et al., 1995a). Indirectly by these Zoo-FISH data, a localization of myogenin can be predicted to porcine chromosome 9q21-qter, which allows preferential selection of polymorphic markers from this respective region.

(f) Association between myogenin genotypes and growth characteristics.

In a first study to determine association between myogenin genotypes and growth characteristics of pigs originating from

populations representing two selection lines of Cofok and Nieuw-Dalland, respectively, the effect of the 3' *MspI* polymorpic site (denoted as "a") on birth weight was determined. The two selection lines differ considerably, the Cofok line has traditionally been selected on homogeneous weights of all piglets in a litter at birth, whereas the Nieuw-Dalland line has not been selected on this trait. For Cofok, 267, and for Nieuw-Dalland, 147 pigs have been typed separately for site a. The number of Cofok animals in the pedigree file was 23092 while of 14687 of those animals the birth weight was known; the number of Nieuw-Dalland animals in the pedigree file with known birth weight was 22569. Typed animals have a chance of 1 for one specific genotype, and thus a chance of 0 for the remaining 2 genotypes. The genotype frequencies of the typed animals were for AA, Aa, and aa 36%, 46% and 18%, respectively, for the Cofok pigs, and 5%, 38%, and 57% for the Nieuw-Dalland pigs. Frequencies for allele A and a were 0.6 and 0.4, respectively, for the Cofok-data, and 0.24 and 0.76 for the Nieuw-Dalland data. For the animals that were not typed, the respective chances for the three genotypes are in between 0 and 1. These chances have been determined with the algorithme developed by Van Arendonk and others (1987) using the allele frequencies, pedigree data and the data from the typed pigs.

The effects of the three genotypes on birth weight were corrected for among others gender, parity of the sow and litter size. Degrees of heretability (h^2) and common environmental components (c^2) were estimated once and incorporated in the model. For birth weight h^2 and c^2 were 0.29 and 0.30, respectively, for the Cofok data; and 0.09 and 0.23, respectively, for the Nieuw-Dalland data.

For the Cofok data, the estimated effects of the genotypes AA or Aa on birth weight were 13.04 g (standard error (SE) 38.79) versus -47.61 g (SE 36.68) (the effects of aa were set on 0 g), for the Nieuw-Dalland data the effects of AA or Aa were 117.89 g (SE 177.20) versus -30.60 g (SE 75.36).

The estimated contrast of birth weights associated with genotype AA or Aa of the Cofok pigs was significantly different

from 0 (60.65 g; SE 27.29); with the Nieuw-Dalland pigs the difference between AA and Aa pigs was 148.49 g (SE 224.25), however, this large difference was not found to be significant, possibly due to the small number of AA pigs typed and the large SE found.

In a second study the effect of the 3' *MspI* polymorpic site (denoted as "a") on birth weight was determined. The two selection lines differ considerably, the Cofok line has traditionally been selected on homogeneous weights of all piglets in a litter at birth, whereas the Nieuw-Dalland line has not been selected on this trait. For Cofok, 606, and for Nieuw-Dalland, 1043 pigs have been typed separately for site a. The number of Cofok animals in the pedigree file was 19148 while of 14295 of those animals a record with fenotypic data was known; the number of Nieuw-Dalland animals in the pedigree file with known fenotypic data was 13244. Typed animals have a chance of 1 for one specific genotype, and thus a chance of 0 for the remaining 2 genotypes. The genotype frequencies of the typed animals were for AA, Aa, and aa 23%, 43% and 34%, respectively, for the Cofok pigs, and 16%, 46%, and 38% for the Nieuw-Dalland pigs. Frequencies for allele A and a were 0.45 and 0,55, respectively, for the Cofok-data, and 0.39 and 0.6 for the Nieuw-Dalland data. For the animals that were not typed, the respective chances for the three genotypes are in between 0 and 1. These chances have been determined with the algorithme developed by Van Arendonk and others (1987) using the allele frequencies, pedigree data and the data from the typed pigs.

The effects of the three genotypes on birth weight were corrected for among others gender, parity of the sow and litter size. Degrees of heretability (h^2) and common environmental components (c^2) were estimated once and incorporated in the model. For birth weight h^2 and c^2 were 0.22 and 0.327, respectively, for the Cofok data; and 0.27 and 0.20, respectively, for the Nieuw-Dalland data.

The estimated contrast of birth weights associated with genotype AA or Aa of the Cofok pigs was significantly different

from 0 (69.7 g; SE 24.5); with the Nieuw-Dalland pigs the difference between AA and Aa pigs was 40.7 g (SE 30.1), however, this large difference was not found to be significant, possibly due to the small number of AA pigs typed and the large SE found. The two independent studies above showed comparable results.

In a third study, it was shown that MyoD-genes influence the onset of muscle development, the meat percentage of offspring is associated with the myf4-polymorphism of their sire.

Boars from a specialized boarline (line OBO) of the DALLAND breeding programme are routinely used in a commercial herd to produce offspring. Each piglet is identified at birth and followed untill it is slaughtered. In total 2303 animals, gilts and boars, were slaughtered during 4 years.

Meat percentage is analysed using the following model:

$$Y = \mu + BV\_BF + MG\_BF + b*S/W + Period + Geno + Sire(Geno) + e$$

where

Y	= Meatpercentage determined by HGP, official Dutch classification
BV_BF	= EBV of the sire for backfat
MG_BF	= probability of the major gene for backfat being AA of the sire
SIW	= linear regression of slaughterweight on meat percentage
Period	= date of slaughter, 8 period of 6 months
Geno	= Genotype of the sire with respect to Myf4 (AA, Aa, aa)
Sire(Geno)	= effect of sire nested within genotype
e	= residual error (variation between animals within sires)

HGP formule combines a measure of backfat and a measure of loin depht, therefore the model contains estimates of backfat, to get a better estimate of the real muscle percentage.

Slaughterweight is known to have a significant effect on meatpercentage.

Sequential sums of squares are calculated for effects in the order as given in the model, using SAS GLM procedure (Type I SS). To test the effect of Myf4-genotype on meatpercentage the variation between genotypes is tested against variation within genotype, i.e. among sires.

Results

All effects in the model were highly significant ($p < .001$). The test of Geno versus Sire(geno) was also significant, $p < 0.0268$. The estimated effect of the Myf4 alleles were:

Genotype	meatpercentage (HGP)
aa	56.74
Aa	56.36
AA	57.26

In addition, for growth parameters no significant effect was found, probably due to the limited amount of data. However, there was a tendency for the heterozygote to be superior.

Given the results mentioned in the third study one can design a breeding programme with specialized lines with e.g. a high or low meat%.

Conclusions

(1) PCR amplification on porcine genomic DNA and screening of a genomic library with human myf cDNA fragments has enabled the isolation and characterization of the pig myogenin gene.

(2) Sequence analysis of a 3.5 kb fragment revealed high similarity in coding and proximal promoter regions between pig, human and mouse myogenin genes.

(3) Three polymorphic *MspI* sites were found in the promoter region, in the second intron and at the 3' side of the gene, respectively. In a panel of 105 unrelated pigs and in several Meishan families, at least 4 myogenin alleles were identified, some of which were breed specific. PCR tests for

the rapid identification of these alleles were developed. This allows association studies with the growth performances of pigs.

(4) Myogenin was assigned to pig chromosome 9.

(5) A statistically significant association has been found between alleles of the 3' *MspI* polymorphic site (site a) and effects on birth weight in a selection line of pigs.

TABLE I: Primer sequences, annealing temperatures and fragment characteristics for PCR amplification reactions of different regions of the pig myogenin gene.

AMPLIFIED REGION (NUCLEOTIDE NRS)	PRIMER SEQUENCES	ANNEALING TEMPERATURE	PREDICTED SIZE OF AMPLIFIED PRODUCT
PCR 1[a] Coding regions and introns 572-2654	FW: M4 5'-GGAATTCCATGGAGCTGTATGARACATC REV:M4R 5'-GGAATTCCAATCTCAGTTGGGCATGGT	61°C	2099 bp
PCR 2[a] EXON 1 572-1005	FW:M4 5'-GGAATTCCATGGAGCTGTATGARACATC REV:M4Rp 5'-TCRCGCTCCTCCTGGTTGA	61°C	442 bp
PCR 3[a] EXON 1-EXON2 988-1858	FW:MMYOGi 5'-CAACCAGGAGGAGCGCGATCTCCG* REV:MMYOGc 5'-AGGCGCTGTGGGAGTTGCATTCACT*	59°C-61°C	871 bp
PCR 4[a] 3'-SIDE	FW:M4P2 5'-TCAGGAAGAACTGAAGGCTG REV:IP12 5'-GTTTCCTGGGGTGTTGC	60°C	353 bp
PCR 5[a] 5'-SIDE 16-1005	FW:M4P3 5'-GAGTCTCATCTGACTGACAC REV:M4Rp 5'-TCRCGCTCCTCCTGGTTGA	60°C	990 bp

Nucleotide numbers are according to the pig genomic sequence in Fig. 1; fw: forward primer; rev: reverse primer; *: primer sequences, previously described by Montarras et al., 1991.

[a] PCR amplifications were performed on genomic DNA and on phage DNA. Genomic DNA was isolated as described (Sambrook et al., 1989) from EDTA-treated blood samples stored at -80°C. Phage DNA was purified from isolated phage clones by the plate lysate method (Sambrook et al, 1989). Myogenin specific PCR amplifications were performed on 50 ng genomic DNA or on 1 µl

of 1:1000 dilutions of phage DNA preparations in 50 µl containing 1.25 U Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) or 0.2 U Super Tth polymerase (SphaeroQ, Leiden, The Netherlands) in the appropriate buffer, 20 pmole of forward and reverse primer, and 0.2 mM of each dNTP (Boehringer Mannheim, Germany). After 5 min of denaturation at 95°C, 35 cycles were carried out of 1 min denaturation at 95°C, 1 to 2 min annealing at the indicated temperature and, depending on the length of the target sequence, 1, 2 or 4 min of elongation at 72°C.

Legend to the Figures

Fig 1: Structure and nucleotide sequence of the pig myogenin gene.

A. Gene structure. Myogenin gene fragments were isolated by PCR amplification on genomic DNA (PCR 1 and PCR 2, Table 1) or by screening of a genomic library with human myf cDNAs. *SacI* fragments isolated from two myogenin containing phage clones (A16 and 17) were subcloned in pBS- and sequenced. Sequence data of the indicated segments were submitted to the EMBL data base and are available under accession numbers X89007, X89209, X89210. B. Nucleotide sequence and deduced amino acid sequence of the pig myogenin gene. Intron sequences are shown in lower case letters; putative TATA box, polyadenylation signal, E-boxes and other regulatory sequences are underlined; arrows denote putative transcription start sites, analogous to human and mouse genomic sequences (Salminen et al., 1991; Edmondson et al, 1992; Yee and Rigby, 1993).

Methods: The sequence was obtained by dideoxy sequencing (Sanger et al., 1977) of (1) PCR fragments amplified on genomic DNA by PCR 1 and 2 (Table 1) and cloned in pUC18, and (2) by analysis of myogenin *SacI* fragments isolated from a genomic library. These fragments were obtained by screening 0.5 x 106 plaque forming Units (pfU) of a porcine genomic library in the

phage vector EMBL3/SP6/T7 (Clontech Laboratories Inc. Palo Alto, CA) by hybridization to the four radioactively labelled human myf cDNA fragments (purchased from ATCC, Rockville, MD; Braun et al. 1989a, 1989b, 1990). After hybridization on duplicate filter lifts (nitrocellulose) for 16 h at 68°C in 6 x SSC, 0.5% (w/v) SDS, 10 mM EDTA, 5 x Denhardt's solution, 100 µg salmon sperm DNA per ml, filters were washed three times for 10 min in 6 x SSC, 0.1% (w/v) SDS and once for 30 min in 2 x SSC, 0.1% (w/v) SDS at room temperature. Thirty seven clones remained positive after three successive rounds of plaque purification. Phage DNA was purified by the plate lysate method (Sambrook et al, 1989) and 17 myogenin positive clones were identified by PCR 1 and PCR 3 amplification (Table 1) on purified phage DNA. Inserts were analysed by XlloI and SacI digestion and Southern (1975) blot hybridization with the myf cDNA probes. SacI fragments of 2 representative clones were subcloned in pBS- and sequenced.

Fig. 2: Detection of a polymorphic *MspI* site at the 3' side of the myogenin gene by Southern RFLP and PCR-RFLP analysis.
A. Southern blot analysis of *MspI* digested genomic DNA of 8 Great Yorkshire (GY) boars, hybridized to a 3' specific myogenin probe (Fig. 1a), revealing a polymorphic fragment (4.2/4.9 kb) as described by Ernst et al. (1993).
B. PCR 4-*MspI* analysis of 8 GY boars. A fragment of 353 bp is amplified (Table 1) and digested with *MspI*, resulting in the 353 bp fragment, corresponding to the 4.9 kb fragment in A, or in 134 bp and 219 bp fragments, corresponding to the 4.2 kb fragment in A.

Methods: Ten µg of porcine genomic DNA, isolated from EDTA treated blood as described (Sambrook et al., 1989), was digested with 100 U *MspI* (Boehringer) following the manufacturer's instructions, for 16 h at 37°C. After precipitation, digested DNA was electrophoresed in a 0.8% (w/v) agarose gel (Sigma, St Louis, MO) and transferred to positively charged nylon membranes (Boehringer Mannheim) by Southern

blotting (Southern, 1975). After fixation of the DNA (30 min, 120°C), membranes were hybridized in a solution containing 5 x SSC, 1% (w/v) blocking solution (Boehringer), 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) SDS, for 16 h at 68°C. Membranes were washed at room temperature three times for 2 min in 2 x SSC, 0.1% (w/v) SDS and twice for 30 min in 0.1 x SSC, 0.1% (w/v) SDS. Hybridizing fragments were visualized by autoradiography at -80°C for 2 days to 1 week.

Table II: Frequency of myogenin genotypes in 7 pig breeds, based on three polymorphic MspI sites.

BREED	NR[b]	PROMOTER[c]			INTRON 2[d]			3'END[c,e]		
		1.8/1.8	1.8/2.3	2.3/2.3	2/2	2/3	2/3	4.2/4.2	4.2/4.9	4.9/4.9
MEISHAN	11	9%	36%	55%	9	36%	55%	0%	0%	100%
PIETRAIN	5	0%	0%	100%	0%	0%	100%	40%	20%	40%
DUROC	10	0%	0%	100%	0%	10%	90%	50%	50%	0%
WILD PIG	10	0%	0%	100%	0%	0%	100%	60%	40%	0%
GYa	40	0%	0%	100%	0%	0%	100%	2.5%	20%	77.5%
DLa	20	0%	0%	100%	0%	10%	90%	30%	40%	30%
HAMPSHIRE	9	0%	0%	100%	0%	0%	100%	22%	56%	22%

[a] GY: Great Yorkshire; DL: Dutch Landrace b number of unrelated pigs tested c Pigs were genotyped by Southern RFLP analysis. The myogenin PCR 1 probe, comprising the complete coding sequences and introns (Fig 1a), detects a 2.3 kb/1.8 kb *MspI* polymorphism in Meishan pigs. DNA treatment and hybridization conditions are as described in Fig. 2. d Pigs were genotyped by PCR 1/*MspI* RFLP analysis, detecting an *MspI* polymorphism in the second intron at position 2394 (Fig. 1b); 2 denotes the absence, 3 the presence of the MspI site at position 2394. ' Pigs were genotyped by (1) Southern RFLP analysis with a 3' specific probe, comprising 195 bp of inton 2 and exon 3 and 4 kb of 3' untranslated sequences (Fig 1a), hybridizing to polymorphic 4.2 kb/4.9 kb MspI restriction fragments, and (2) PCR 41MspI RFLP analysis, visualizing the same MspIpolymorphism at the 3' side of the gene. Experimental conditions were as described in Table I and Fig. 2.

Fig. 3: Mendelian segregation of two *Mspl* polymorphisms at the pig myogenin gene locus in a two generation family of Meishan pigs (square denotes sire, circle denotes dam).

A. Southern blot analysis of *MspI* digested genomic DNA, hybridized to the myogenin PCR 1 fragment (Table I, Fig. 1), visualizing 2.3/1.8 kb polymorphic *MspI* fragments. Square denotes sire; circle denotes dam. DNA treatments and hybridization conditions are as described in Fig. 2.

B. PCR 1/*MspI* analysis (Table I), detecting an *MspI* polymorphism in the second intron of the myogenin gene at position 2394; the amplified fragment of 2099 bp is digested by *MspI*, resulting in 4 fragments of 1505, 171, 155 and 268 bp or in 3 fragments of 1505, 171 and 423 bp.

C. PCR 5 analysis (Table I), showing the presence of the 2.3 kb allele by amplification of a fragment of 990 bp.

Fig. 4: Schematic representation of the 4 myogenin alleles that were distinguished in the pig breeds and crosses tested, based on the different observed combinations of three polymorphic MspI sites at this gene locus. The polymorphic sites are indicated by a, b, c?.

TABLE III: Statistical analysis for concordance and correlation of myogenin with pig chromosomes

CHROMOSOME/ REFERENCE LOCI[a]	con[b]	ø[c]
1/IFNA	67	0.41
2/S0091	67	0.41
3/APOB	81	0.63
4/S0001	52	0.04
5/S0092	67	0.36
6/RYR1	62	0.35
7/TNFB	76	0.51
8/ALB	71	0.37
9/S0095	90	0.81
10/S0038	81	0.60
11/S0009	52	0.04
12/GH	81	0.59
13/S0076	43	-0.16
14/DAO	48	0.01
15/S0088	38	-0.23
16/S0006	52	-0.01
17/ENDO	62	0.28
18/S0062	52	0.14
X/S0022	46	-0.08
Y/SRY	62	0.44

[a] Establishment and cytogenetic characterization by QFQ-banding and FISH of 21 porcine x rodent somatic cell hybrids as well as analysis by PCR has been described earlier in detail (Rettenberger et al., 1994a, 1994b, 1994c, 1995b).

[b,c] Statistical evaluation for concordance and correlation coëfficient φ has been performed according to Chevalet and Corpet (1986). The distribution pattern of myogenin was compared with the distribution pattern of the reference loci in each hybrid. Concordance corresponds to the percentage of hybrids which are identical for the presence/absence of the reference locus and myogenin. A marker is syntenic with a reference locus if the correlation coëfficient φ is >0.74 for n=20 hybrid lines, with a probability of 2.5% of making an incorrect decision; if φ <0.59, synteny can be excluded. The values for chromosome 9 where myogenin maps to, are typed in bold.

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CLAIMS

1. An isolated or recombinant pig myogenin gene specific nucleic acid molecule or pig myogenin gene specific fragments thereof comprising or hybridising to the nucleotide sequence of fig. 1 or its complementary sequence or the RNA equivalents thereof.

2. A method localising, identifying or marking genes or alleles or quantitative trait loci of pigs, using a molecule or a fragment or fragments thereof according to claim 1

3. A method according to claim 2 whereby genes or alleles or quantitative trait loci are localised, identified or marked that are associated with production traits of pigs

4. A method according to claim 2 or 3 to identify or mark alleles of the pig myogenin gene,

5. A method according to claim 4 distinguishing between alleles of the myogenin gene of pigs.

6. A method according to claim 5 by detecting specific restriction sites in an allele of the myogenin gene of pigs.

7. A method according to claim 6 whereby an *MspI* restriction site is detected.

8. A method localising, identifying or marking genes or alleles or quantitative trait loci in samples, in particular biological samples, cells or tissues, such as but not limited to hair, skin or blood, of pigs, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci of pigs

9. A diagnostic assay or kit according to claim 8.

10. A method according to anyone of claims 2-7 localising, identifying or marking genes or alleles or quantitative trait loci in samples, in particular biological samples, cells or tissues, such as but not excluded to hair, skin or blood of pigs, by allowing for specific amplification of genomic fragments of those genes or alleles or quantitative trait loci,

11. A method according to anyone of claims 8 or 10 in which the method of amplification is the polymerase chain reaction.

12. Diagnostic assay or kit according to claim 11.

13. A method according to anyone of claims 2-12 identifying differences between alleles of the pig that are associated with differences in production traits of pigs

14. A method according to claim 13 identifying alleles of the pig that are associated with improved production traits of pig

15. Use of the methods according to anyone of claims 2-14 in marker assisted identification of pigs or in marker assisted selection of pigs.

16. Use of the methods according to claims 14 or 15 in breeding programmes

Figure 1b

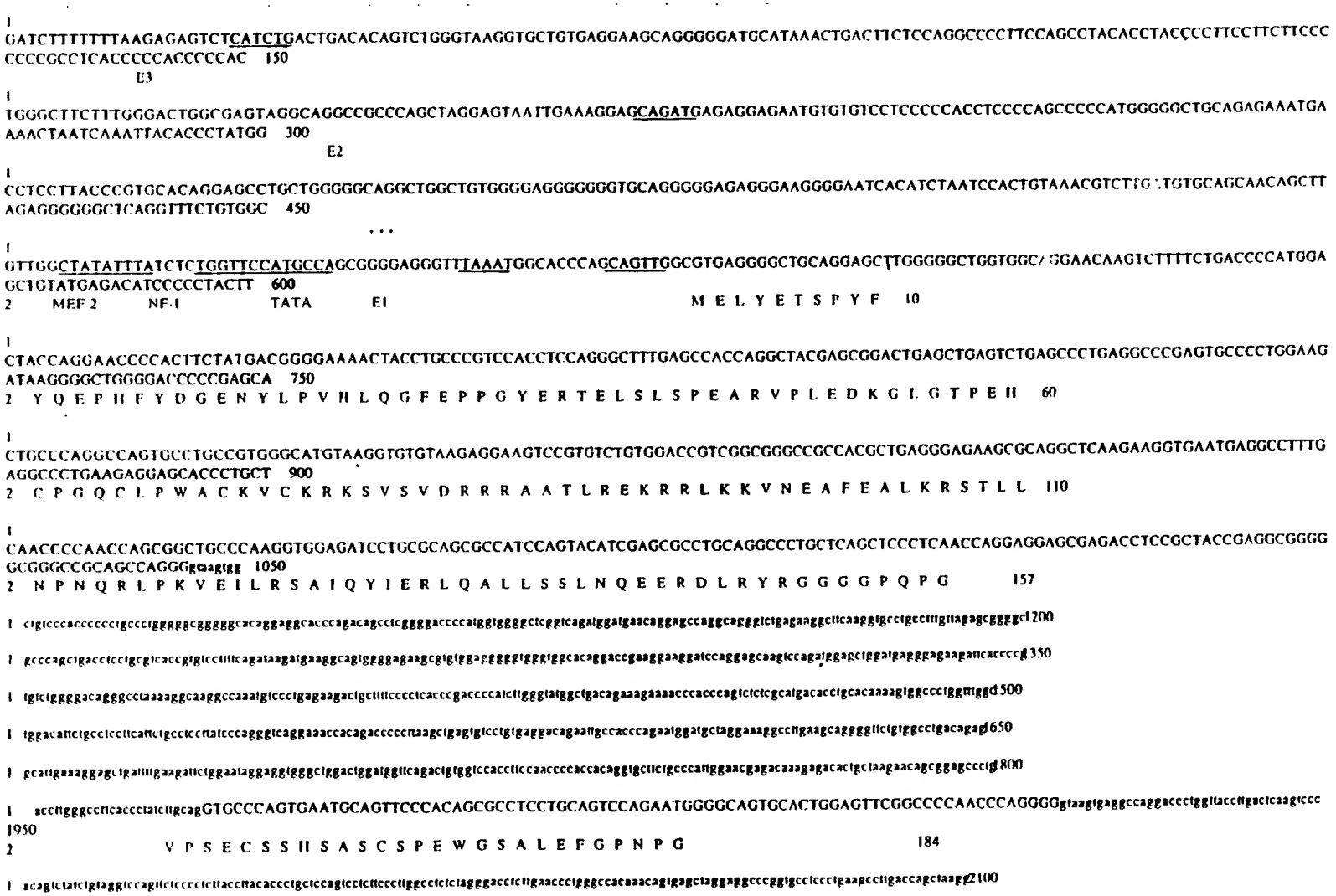

Figure 1b continued

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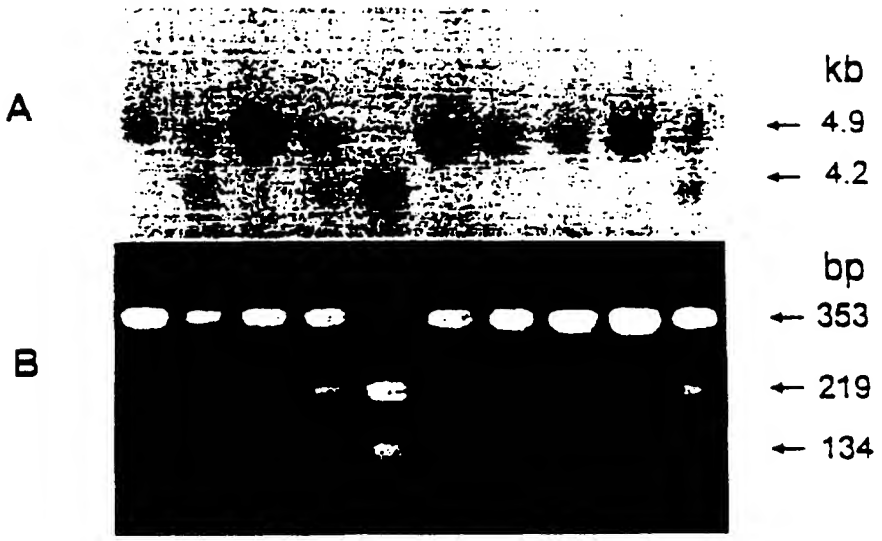

Fig. 2

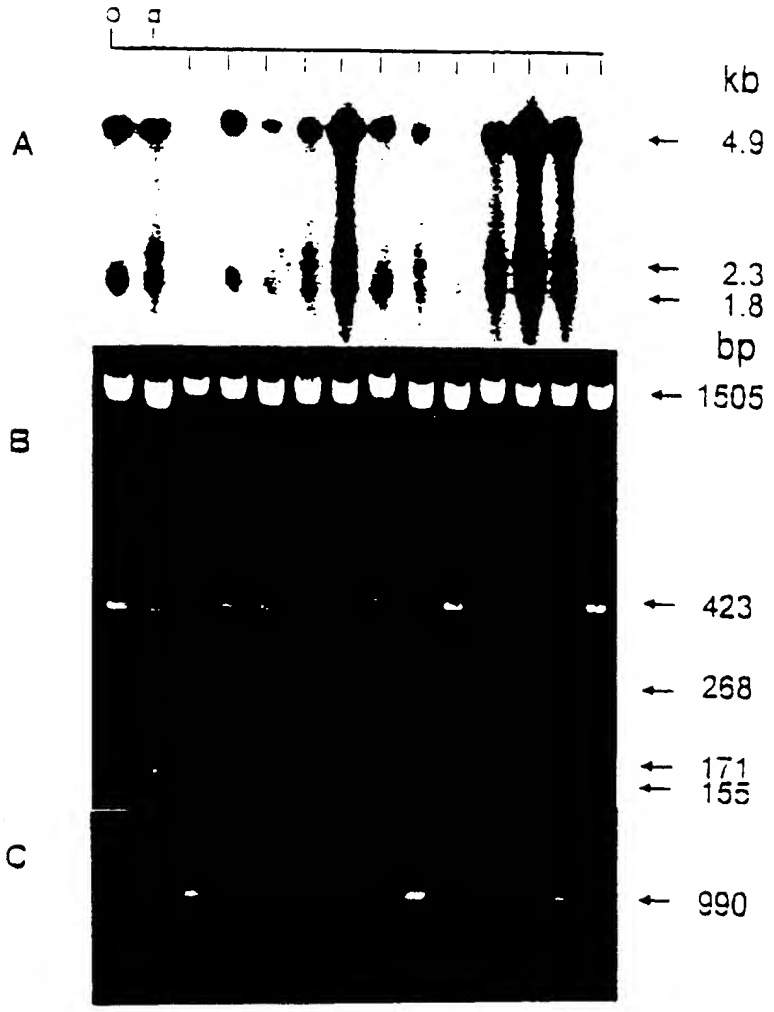

Fig. 3

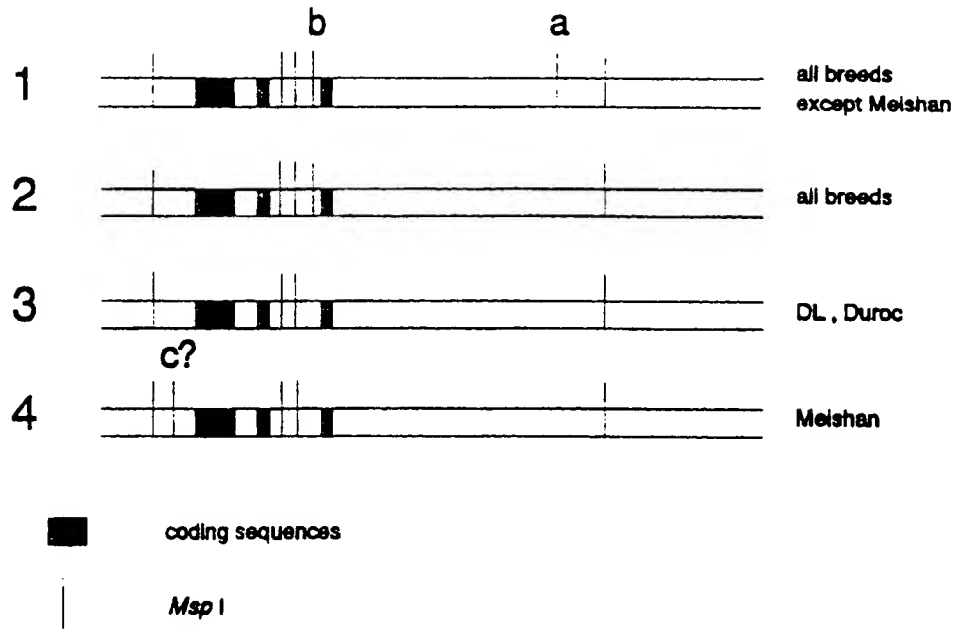
b
a
1
all breeds
except Meishan
2
all breeds
3
DL , Duroc
c?
4
Meishan
coding sequences
Msp I

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 96/00497

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANIMAL BIOTECHNOLOGY, vol. 6, no. 2, October 1995, NEW YORK US, pages 79-92, XP000196214 G.P. BRILEY ET AL.: "Cloning and expression of the porcine myogenin gene"	1
Y	see the whole document ---	2-16
X	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, vol. 0, no. 18d, 1994, US, page 522 XP000196220 A SOUMILLION ET AL.: "Isolation and characterization of porcine MYF genes" * Abstract No. W358 * ---	1
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 March 1997	04. 04. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	De Kok, A

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J ANIMAL BREEDING GENETICS, vol. 111, 1994, BERLIN DE, pages 404-412, XP000196251 M.F.W. TE PAS ET AL.: "Genetic regulation of meat production by embryonic muscle formation - a review" cited in the application see the whole document	2-16
A	JOURNAL OF ANIMAL SCIENCE, vol. 71, 1993, CHAMPAIN US, page 3479 XP000196219 C.W. ERNST ET AL.: "Rapid communication: MspI restriction fragment length polymorphism at the swine myogenin locus" cited in the application see the whole document	1-7
A	WO 92 18651 A (IOWA STATE UNIVERSITY RESEARCH FOUNDATION, INC.) 29 October 1992 see the whole document	1-16

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International application No.
PCT/NL 96/00497

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 8,9
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Claims searched incompletely because of lack of technical disclosure. (Art. 17(2)(b)).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 96/00497

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9218651 A	29-10-92	AU 660994 B	13-07-95
		AU 1917692 A	17-11-92
		BR 9205918 A	08-11-94
		CA 2108579 A	20-10-92
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		JP 6506837 T	04-08-94
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